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Two international Round Robin studies showed good comparability of 5-methyltetrahydrofolate, but poor comparability of folic acid measured in serum by different HPLC-MS/MS methods

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Abstract

Background—Serum folate methods produce different results. The comparability of HPLC-MS/MS methods is not well-documented.

Objective—We conducted an international "Round Robin" investigation to assess the comparability, precision, and accuracy of serum folate HPLC-MS/MS methods.

Design—The CDC laboratory, 7 laboratories with independently-developed methods (group 1), and 6 laboratories with an adapted CDC method (group 2) analyzed folate forms in 6 serum pools and 6 calibrators from CDC (duplicate analysis over 2 days) and in 2 three-level reference materials (duplicate analysis).

Results—All laboratories measured 5-methyltetrahydrofolate (5-methylTHF) and folic acid; some measured additional folate forms. Geometric mean concentrations (nmol/L) for 5-methylTHF in the 6 serum pools were 18.3 (CDC), 13.8–28.9 (group 1), and 16.8–18.6 (group 2); for folic acid, 3.42 (CDC), 1.09–4.74 (group 1), and 1.74–2.90 (group 2). The median imprecision (CV) for 5-methylTHF was 4.1% (CDC), 4.6%–11% (group 1), and 1.7%–6.0% (group 2); for folic acid, 6.9% (CDC), 4.9%–20% (group 1), and 3.9%–23% (group 2). The mean (SD; range) recovery of 5-methylTHF spiked into serum was 98% (27%; 59%–138%) for group 1 and 98% (10%; 82%–111%) for group 2; for folic acid, 93% (29%; 67%–198%) for group 1 and 81% (16%; 64%–102%) for group 2. The mean relative bias for 5-methylTHF compared to the reference material certificate value was 12% (CDC), -24% to 30% (group 1), and -0.6% to 16% (group 2); for folic acid, 73% (CDC), -47% to 578% (group 1), and -3.3% to 67% (group 2).

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Supplemental Tables 1–8, Supplemental Figures 1–3, and Supplemental Text 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

Conclusions—For 5-methylTHF, group 2 laboratories demonstrated better agreement and precision, less variable spiking recovery, and less bias using a reference material. Laboratory performance for folic acid was highly variable and needs improvement. Certified reference materials for serum folate forms and total folate are needed to improve method accuracy.

Keywords

MeFox; tetrahydrofolate; 5-formyltetrahydrofolate; 5,10-methenyltetrahydrofolate

INTRODUCTION

Serum folate is an important biomarker to assess short-term folate status [1]. Previous studies focused on serum total folate and showed poor method agreement [2-5]. Little is known about the comparability of chromatography-based methods that measure individual folate forms, yet variation in biomarker concentrations across laboratories has to be understood to meaningfully compare data from different laboratories. While chromatography-based methods require complex sample preparation, they provide a high degree of specificity and often also high sensitivity and precision, particularly when HPLC is coupled to a tandem-mass spectrometer [5]. HPLC-MS/MS methods that use stable isotope-labeled internal standards are considered higher-order methods that offer a high degree of accuracy. Over the years mass spectrometers have become smaller in foot-print, less expensive, more robust, and thus more available to specialized reference, research, clinical, and public health laboratories. However, the comparability and performance of HPLC-MS/MS methods for serum folate forms has not yet been assessed systematically.

Several laboratories have developed isotope-dilution HPLC-MS/MS methods to quantitate serum folate forms [6-20]. The methods differ in how many folate forms are measured, how folate is extracted from the sample, what chromatography and instrumentation is used, and how the assay is calibrated. The Nutritional Biomarkers Laboratory at the Centers for Disease Control and Prevention (CDC) has successively expanded and improved its originally published method [6] to include folate forms beyond the 2 main circulating forms of 5-methyltetrahydrofolate (5-methylTHF)[7] and folic acid, to automate the solid-phase extraction step to an 8-probe and later 96-probe system, to reduce the required specimen volume from 275 to 150 μL, and to achieve separation of 2 isobaric compounds, 5-formyltetrahydrofolate (5-formylTHF) and a pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox) [7-9]. Over the years, the CDC laboratory also worked with scientists from several research and public health laboratories who adapted the CDC method.

To generate much needed data on HPLC-MS/MS method comparability and performance, the CDC laboratory conducted 2 Round Robin studies with laboratories who used independently-developed serum folate methods and with laboratories who adapted the CDC method. The main objective of these studies was to investigate how comparable, precise, and

[7]Abbreviations: 5-formylTHF, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; GCV, geometric coefficient of variation; ICC, intraclass correlation coefficient; LOD, limit of detection; NIST, National Institute of Standards and Technology; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RC, repeatability coefficient; SRM, Standard Reference Material; THF, tetrahydrofolate

accurate serum folate results were when generated by different methods in different laboratories. A secondary objective was to assess differences in calibrators used across laboratories.

SUBJECTS AND METHODS

Participating laboratories

In 2015, CDC conducted 2 international Round Robin method comparison studies: for laboratories who used independently-developed HPLC-MS/MS serum folate methods (group 1) and for laboratories who previously adapted a CDC HPLC-MS/MS method (group 2). CDC invited 8 laboratories per group.

Samples

CDC provided each laboratory with 6 serum pools (2 sets for analysis over 2 days), 6 folate calibrators (2 sets for analysis over 2 days, plus a back-up set for an unplanned repeat analysis), and 2 three-level reference materials (1 set for analysis on 1 day). The sample IDs for the serum pools were blinded and the 2 sets were boxed in a different sequence. Each vial contained 1 mL serum. Serum pools 5 and 6 were the same base material; serum pool 6 was spiked with each folate calibrator to assess recovery compared to the unspiked serum pool (10 nmol/L of 5-methylTHF and 5 nmol/L each of folic acid, MeFox, 5-formylTHF, tetrahydrofolate [THF], and 5,10-methenyltetrahydrofolate [5,10-methenylTHF]). Each serum pool was prepared by CDC from human serum obtained from anonymous blood donors (Tennessee Blood Services, Memphis, TN). The CDC individual folate calibrators had a concentration of 100 nmol/L in 0.1% ascorbic acid (1.0 mL/vial for 5-methylTHF and 0.5 mL/vial for other folate forms). A National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1955 (3 levels; 1 mL/vial) was included because this reference material has certified values for 5-methylTHF and reference values for folic acid [21]; NIST SRM 3949 (3 levels; 1 mL/vial) is a new material under development. Samples were stored at -70°C when not in use and shipped to laboratories on dry ice. Laboratories acknowledged the receipt of the shipment and its condition.

Laboratory analysis

CDC provided each laboratory with detailed instructions on how to analyze the samples. Each CDC folate calibrator was diluted to 20 nmol/L for 5-methylTHF (1:5 dilution) and 5 nmol/L for other folate forms (1:20 dilution) and analyzed as an unknown sample. A mixed calibrator was prepared (same concentrations) and analyzed as an unknown sample. The serum pools and CDC calibrators were analyzed in duplicate over a period of 2 days (4 independent measurements), while the reference materials were analyzed in duplicate on 1 day (2 independent measurements). CDC provided each laboratory with a customized report template containing their individual sample IDs and requested that laboratories report the measured folate concentrations in nmol/L and provide the method limit of detection (LOD), the method reference or a short method description, and information on calibrators and internal standards. All laboratories used their in-house materials and protocols (e.g., calibrators, QC, reagents, consumables and instrumentation). The CDC laboratory also analyzed all study samples according to the same instructions.

Statistical analysis

The study was designed to compare serum folate concentrations among laboratories as well as the repeatability of the methods used by each laboratory. Data in tables and figures are presented in the following sequence: CDC laboratory, group 1 laboratories, group 2 laboratories. We opted not to calculate all-lab-means, as these estimates are influenced by outliers. We calculated for each laboratory and each serum pool, calibrator (individual and mixed), and reference material the mean concentration, SD, and CV of the 4 measurement results (2 results for reference materials). We used the median CV across the 6 serum pools as a measure of imprecision for each laboratory. We used a 2-way random effects model with restricted maximum likelihood estimation to partition the total variance of each laboratory method into between-sample variance and analytic variance, composed of between-day and within-day variance. The dependent variables, 5-methylTHF, folic acid, and MeFox concentrations, were log transformed. The random effects in the model were sample (6 serum pools) and day (day 1 and day 2). The model provided estimates of the geometric mean, the geometric coefficient of variation (GCV) for each error component, and the percent variance relative to the total variance for each laboratory. We calculated the repeatability coefficient (RC) for each laboratory ($RC = \sqrt{2} * 1.96 * SD_{log}$; SD_{log} was the within-day SD estimated from the model) and reported the anti-log of the repeatability coefficient, r_c. On the log scale, the r_c is interpreted as the 95% range for the ratio between 2 replicates on the same day ($\times / \div e^{RC}$). We determined accuracy by assessing spiking recovery for each folate form spiked into serum pool 6 relative to the unspiked serum pool 5. Recovery was calculated as {[(measured spiked sample – measured unspiked sample)/spike] * 100} and zero was used for the unspiked sample if the concentration was <LOD. We used the mean (SD) spiking recovery as a measure of accuracy for each laboratory and folate form. For 5-methylTHF and folic acid, we also determined accuracy using NIST SRM 1955 by calculating the relative bias for each measurement result compared to the certificate value and then calculating the mean relative bias.

RESULTS

Participating laboratories

The participating laboratories were from 8 countries and used various sample extraction and clean-up methods as well as different chromatography and instrumentation to measure serum folate forms by HPLC-MS/MS (Table 1). All laboratories used reversed-phase chromatography at acidic pH and electrospray ionization in positive ion mode. Seven laboratories in group 1 (#1–#7) and 6 in group 2 (#11–#16) reported results in addition to the CDC laboratory (#10). All laboratories measured the 2 main folate forms 5-methylTHF and folic acid, while some laboratories, including the CDC laboratory, also measured other folate forms. Calibration ranges and LODs varied by folate form and laboratory; generally, calibration ranges did not exceed 100 nmol/L and LODs were <1 nmol/L (Supplemental Table 1). Most laboratories used compound-specific $^{13}C_5$-analogues as internal standards, however 2 laboratories (#3 and #4) used deuterated internal standards (Supplemental Table 1). These 2 laboratories also measured their calibrators directly without carrying them through the sample extraction process (Table 1). With few exceptions, most laboratories assigned concentrations to their folate calibrators spectrophotometrically (Supplemental

Table 2). Consistent molar absorptivity coefficients were used for 5-methylTHF, folic acid (except laboratory #1), and 5-formylTHF, but not for THF, 5,10-methenylTHF, and MeFox (Supplemental Table 2).

Agreement among laboratories

Based on the individual measurement results for the 6 serum pools, we observed differences in repeatability by sample and laboratory and the variance increased with increasing concentration. Visual inspection showed that for 5-methylTHF, the repeatability was poor for laboratories #3 and #4 and the agreement among laboratories was generally good, particularly in group 2 (Supplemental Figure 1). For folic acid, the repeatability was poor for laboratories #3 and #4; the agreement among laboratories was poor for pools 4–6 (Supplemental Figure 2). For MeFox, the repeatability was poor for several laboratories, but especially for laboratories #14 and #16; the agreement among laboratories was also poor (Supplemental Figure 3).

We used the mean of the 4 measurement results for each of the 6 serum pools to further assess agreement among laboratories and calculated the group 1 and group 2 mean (SD) for each serum pool. The 5-methylTHF concentration ranges were similar across the groups (~9–38 nmol/L) (Table 2). While the grand mean among pools was similar for group 1 and group 2, the average SD among pools was higher for group 1 compared to group 2 (mean [SD]: 21.1 [5.2] and 20.1 [0.8] nmol/L, respectively). Concentrations of 5-methylTHF were similar across laboratories except for laboratory #3, which measured higher and had the widest range of results (Figure 1, panel A). The folic acid concentration ranges were also similar across the groups (~1–14 nmol/L) (Table 3). As seen with 5-methylTHF, the grand mean among pools was similar for group 1 and group 2, but the average SD was higher for group 1 (3.95 [1.75] and 3.72 [0.58] nmol/L, respectively). Folic acid concentrations varied across laboratories and laboratory #4 measured consistently higher and had the widest range of results (Figure 1, panel B). The MeFox concentration ranges were also similar across the groups (~2.5–14 nmol/L) (Table 4). The grand mean and the average SD among pools were similar in both groups (7.03 [1.62] and 8.42 [1.53] nmol/L). MeFox concentrations varied across laboratories and laboratory #16 had the widest range of results (Figure 1, panel C).

The 3 minor folate forms (5-formylTHF, THF and 5,10-methenylTHF) measured in a spiked serum pool generally showed reasonable agreement among laboratories with some exceptions: laboratories #15 and #16 measured higher for 5-formylTHF, laboratory #4 did not detect any THF, and laboratory #16 measured higher for THF (Table 5).

Imprecision

The imprecision (CV) for the 6 serum pools varied by laboratory and analyte (Figure 2). The widest CV ranges were obtained by laboratories #4 and #5 for 5-methylTHF (panel A), laboratories #3, #4, #5, #11, and #14 for folic acid (panel B), and laboratories #14 and #16 for MeFox (panel C). The median CV for 5-methylTHF was 4.1% (laboratory #10), 4.6%–11% (group 1), and 1.7%–6.0% (group 2) (Table 2). For folic acid, the median CV was 6.9% (laboratory #10), 4.9%–20% (group 1), and 3.9%–23% (group 2) (Table 3). For MeFox, the median CV was 5.6% (laboratory #10), 3.7%–5.1% (group 1), and 4.5%–30% (group 2)

(Table 4). The CV for the 3 minor folate forms in a spiked serum pool was generally ≤10% with some exceptions: laboratories #2 and #3 for 5-formylTHF, laboratory #3 for THF, and laboratories #6 and #16 for 5,10-methenylTHF (Table 5). In general, the imprecision estimates were comparable or higher than estimates published by group 1 laboratories (Supplemental Table 3).

Sources of variation

We assessed the magnitude of variation between samples (serum pools) and between measurements (between-day and within-day) of the same sample relative to the total variation (Table 6). The between-sample variance explained over 90% of the total variance for each laboratory. This parameter is also known as the intraclass correlation coefficient (ICC). For 5-methylTHF, the ICC was >97% for 11 of 14 laboratories (except for laboratories #3, #4 [lowest ICC], and #6). The between-sample GCV was similar across laboratories (51.3%–65.5%) except for laboratory #5 (80%). For folic acid, the ICC was >97% for 10 of 14 laboratories (except for laboratories #3, #4, #11 [lowest ICC], and #14). The between-sample GCV was similar across laboratories (98.6%–163%) except for laboratories #1, #2, and #5. For MeFox, the ICC was >98% for 6 of 8 laboratories (except for laboratories #14 and #16 [lowest ICC]). The between-sample GCV was fairly similar across laboratories (47.6%–75.4%).

The between-day and within-day variances made up a small percentage of the total variance. For 5-methylTHF, the within-day variance generally exceeded the between-day variance. Only 2 laboratories (#10 and #6) had a higher between-day than within-day variance. For 8 of 14 laboratories the between-day variance was estimated to be zero and 5 of these 8 laboratories were from group 2. For folic acid and MeFox, the contributions of between-day and within-day variances to the total variance were similar, albeit the within-day variance generally exceeded the between-day variance (except for laboratories #4 and #11 for folic acid).

We observed differences in the repeatability of replicates within a day across laboratories and analytes. A low r_c is desirable, as it demonstrates higher repeatability between pairs of measurements for a given analyte. Laboratories #3 and #4 had the highest r_c of approximately 1.3 for 5-methylTHF. This value can be used to construct a 95% range for the ratio between 2 replicates on the same day as (1.3^{-1}, 1.3); in other words, 95% of the ratio of pairs of replicates is expected to fall between 0.77 and 1.3. For folic acid, laboratories #3, #5, #11 and #14 (highest r_c) all had an r_c >1.5. For MeFox, laboratories #14 and #16 had the highest r_c. On average, folic acid had the worst relative repeatability compared to 5-methylTHF and MeFox, though laboratory #16 had an r_c for MeFox (2.27) that was higher than any other reported r_c.

Accuracy

The spiking recovery of 5-methylTHF varied by laboratory (Table 7), with group 1 (mean [SD], range: 98% [27%], 59%–138%) reporting higher variation than group 2 (98% [10%], 82%–111%). Laboratory #10, laboratories #1 and #5 (group 1), and laboratories #13 to #16 (group 2) achieved nearly complete spiking recovery (100% ± 10%). The spiking recovery

of folic acid also varied by laboratory (group 1: 93% [47%], 67%–198%; group 2: 81% [14%], 64%–102%), but was incomplete for most laboratories. The spiking recovery of MeFox was nearly complete for laboratories #7, #10, #13, and #16. Spiking recoveries for the 3 minor folate forms were quite different by laboratory. Some laboratories obtained highly unusual recoveries for 5-formylTHF (#15 and #16) and for THF (#4 and #16). Spiking recoveries were mostly comparable, but some were lower or higher than estimates published by group 1 laboratories (Supplemental Table 3).

We evaluated accuracy for 5-methylTHF and PGA using NIST SRM 1955 (Figure 3). Laboratory #3 had the largest positive bias and widest bias range compared to the reference material certificate value for both 5-methylTHF (panel A) and folic acid (panel B). The mean relative bias was 12% (laboratory #10), -24% to 30% (group 1), and -0.6% to 16% (group 2) for 5-methylTHF; and 73% (laboratory #10), -47% to 578% (group 1), and -3.3% to 67% (group 2) for folic acid (Table 8). While NIST SRM 1955 cannot be used to assess accuracy of MeFox, we observed similar concentrations among laboratories (Supplemental Table 4). Results for the 3 minor folate forms were nearly all <LOD and are not presented. Results for a NIST reference material under development (SRM 3949) also showed similar 5-methylTHF concentrations among laboratories, but variable folic acid concentrations (Supplemental Table 5). Results for MeFox (levels 1–3) and for the 3 minor forms (level 3) were generally similar with some exceptions: laboratory #16 measured lower for MeFox, laboratory #4 measured lower for 5-formylTHF, and laboratories #15 and #16 measured higher for 5-formylTHF (Supplemental Table 6). Results for the 3 minor folate forms for levels 1–2 are not presented (mostly <LOD).

CDC folate calibrators

We found good agreement among laboratories for the CDC 5-methylTHF calibrator, measured individually (single) or in a mixture with other calibrators (mix), but much poorer agreement for the other folate forms (Figure 3 and Supplemental Table 7). While the mean concentrations for the 3 main analytes were similar for group 1 and group 2, the SD was higher for group 1: 5-methylTHF (target: 20 nmol/L): mean (SD), 20.5 (1.84) *vs.* 19.8 (0.62) nmol/L; folic acid: (target: 5 nmol/L): 3.83 (1.61) *vs.* 3.36 (0.83) nmol/L; MeFox (target: 5 nmol/L): 4.56 (1.12) *vs.* 5.16 (0.24) nmol/L. For the 3 minor folate forms, the agreement among laboratories was lower and the SD was higher compared to the 3 major folate forms. Some notable abnormal results were obtained for 5-formylTHF (laboratory #15) and for THF (laboratories #4 and #16). For some laboratories we noticed differences between the single calibrator and the mixed calibrator for 5-formylTHF and 5,10-methenylTHF, which can be due to pH dependent folate interconversions. When we calculated non-methylfolate (sum of 5-formylTHF, THF, and 5,10-methenylTHF) in the mixed calibrator for the 9 laboratories that measured these 3 folate forms, 5 laboratories (#5, #7, #10, #13, and #14) obtained results within 1 nmol/L of the target value of 15 nmol/L, while laboratories #4 (9.4 nmol/L) and #6 (11.7 nmol/L) underestimated and laboratories #15 (22.4 nmol/L) and #16 (28.3 nmol/L) overestimated the target value (Supplemental Table 7).

DISCUSSION

The 2 current international studies for independently-developed (group 1) and adapted CDC methods (group 2) provide results for 13 laboratories plus the CDC laboratory and are to our knowledge the first investigation where comparability, precision, and accuracy for serum folate HPLC-MS/MS methods were systematically assessed. The laboratory comparability was good for 5-methylTHF, but poor for folic acid and MeFox. Given the higher 5-methylTHF serum concentrations compared to other folate forms, it was not surprising that precision and accuracy were best for this compound. However, we noted differences in method performance among laboratories and generally better performance in group 2 than in group 1 laboratories.

NIST reference materials (SRM 1955 and SRM 1950) with certified 5-methylTHF concentrations have been available for years, which may be another reason why we obtained the best performance for this compound. SRM 1955 was value assigned in 2004 by 4 independent NIST methods and the CDC method [23]. Twelve of 14 laboratories agreed within ±20% of the certified values for SRM 1955. Laboratories #5 (-24%) and #3 (30%) deviated most from certificate values. These 2 laboratories also had the highest mean difference from the CDC laboratory in serum pools (-23% and 59%, respectively), while other laboratories agreed within ±10% (Supplemental Table 8). The discrepancy for laboratory #5 does not appear to be calibration related, because of their close agreement with the target value for the 5-methylTHF calibrator (19.6 *vs.* 20 nmol/L) and their complete spiking recovery (100%). Laboratory #3 also showed reasonably close agreement with the target value for the 5-methylTHF calibrator (23.2 *vs.* 20 nmol/L), but the laboratory displayed high imprecision (median CV 9.8%) and the lowest and most variable spiking recovery (mean [SD]: 59% [68%]). This indicates different method performance for serum samples and calibrators, possibly because the laboratory did not carry the calibrators through the sample extraction process.

We observed larger imprecision for folic acid in serum samples with lower (~1 nmol/L) compared to higher folic acid concentration. Laboratories #3 and #4 (both used d_4-folic acid and did not carry the calibrators through sample extraction) obtained the highest results for most serum pools, for SRM 1955 (mean bias: 578% and 236%, respectively), and for the folic acid calibrator (6.0 and 6.21 nmol/L, respectively) and showed high imprecision (median CV: 20% and 19%, respectively). Other laboratories obtained lower folic acid results compared to the CDC laboratory for the serum pools (-63% to -13%; Supplemental Table 7) and for the folic acid calibrator (2.32–4.55 *vs.* 5 nmol/L), and most obtained incomplete spiking recoveries (64%–102%). This was unexpected and necessitated a thorough investigation.

CDC conducted experiments that compared folic acid primary stock solutions of variable concentrations and age, folic acid intermediate stock solutions prepared in water *vs.* 0.1% ascorbic acid, and buffering of the daily calibrator mixture *vs.* using 0.1% ascorbic acid as the diluent. We found problems with folic acid solubility at certain pH and concentration conditions (for further details, consult Supplemental Text 1). As a result, the folic acid calibrator value was incorrectly assigned (~30% too high) leading to an overestimation of

serum concentrations. After correcting the calibration bias, CDC obtained on average 34% lower results for the serum pools (Table 3). The new folic acid values for the NIST SRM 1955 (levels 1, 2, and 3: 0.54, 1.17, and 1.14 nmol/L, respectively) were on average 36% lower (Table 8) and in good agreement with the certificate reference values (0.49, 1.05, and 1.07 nmol/L, respectively). Only 5 laboratories measured lower than the CDC laboratory for the serum pools after CDC corrected the calibration bias *vs.* 12 laboratories before (Supplemental Table 7), but the agreement among laboratories was still poor (-43% lower to 125% higher than the CDC laboratory). The larger imprecision observed for folic acid and the large differences in mean relative bias for NIST SRM 1955 (-47% to 578%) among laboratories raise the question whether folic acid measurement may be affected by solubility issues in other laboratories as well.

Fewer laboratories measured MeFox and most obtained mean results for the 6 serum pools within ±15% of the CDC laboratory, except for laboratories #6 (32% lower) and #16 (26% higher) (Supplemental Table 7). Laboratory #6 obtained the lowest spiking recovery (66%) and the lowest concentration for the MeFox calibrator (3.33 *vs.* 5 nmol/L), possibly indicating a calibration bias. Laboratory #16 showed the highest imprecision (median CV 30%), but obtained reasonable spiking recovery (90%) and measured close to the target value (5.23 *vs.* 5 nmol/L) for the MeFox calibrator. This may be indicative of sample processing issues that lead to large variability.

This study only allowed limited interpretation of results for the 3 minor folate forms because concentrations were <LOD in most serum samples and fewer laboratories measured these compounds. Nonetheless, some useful information was gained from the spiked serum pool and from the calibrators. Laboratory #15 obtained 148% higher 5-formylTHF results in the serum pool than the CDC laboratory (Supplemental Table 7), measured higher than the target (11.6 *vs.* 5 nmol/L) for the 5-formylTHF calibrator, and obtained a spiking recovery of 259%, indicating a potential calibration bias or interference. Similarly, laboratory #16 obtained 111% higher 5-formylTHF and 345% higher THF results than the CDC laboratory for the serum pool (Supplemental Table 7), measured higher than the target (8.75 *vs.* 5 nmol/L for 5-formylTHF; 14.0 *vs.* 5 nmol/L for THF) for the calibrator, and obtained a spiking recovery of 221% for 5-formylTHF and 460% for THF, indicating a potential calibration bias or interference for both compounds. Laboratory #4 did not detect any THF in the spiked serum pool or in the THF calibrator, and obtained a spiking recovery of 0% for THF and 198% for folic acid. This may indicate that THF was lost during sample preparation and partially oxidized to folic acid, possibly during the heat extraction. Laboratory #3 showed the highest imprecision for 5-formylTHF (22%) and THF (38%), which could again be related to sample processing.

The results of the random effects models confirmed many of the observations made from figures and descriptive statistics. However, some aspects such as the independent and unbiased estimation of the 3 sources of variability can only be elucidated by a statistical model. The model results showed that for most laboratories the between-day variance was smaller than the within-day variance. Specifically, for 5-methylTHF, the model estimated a zero between-day GCV for half of the laboratories. While intuitively unappealing, as one expects observations within days to be more correlated than between days, this type of

observation can be a characteristic of data. We confirmed that the average percent change in the absolute difference of the geometric means between day 1 and day 2 was smaller for laboratories with an estimated zero between-day GCV (1.0%, 1.1%, and 1.6% for 5-methylTHF, MeFox, and folic acid, respectively) compared to the remaining laboratories (7.3%, 7.3%, and 18.0%, respectively). Laboratory #6 had the largest between-day variance for 5-methylTHF (10.9%), which seemed to have been caused by variability in the calibration curve parameters. The models further confirmed that group 1 and group 2 laboratories seemed to achieve similar results in imprecision across the analytes and also identified more issues with the reproducibility for folic acid compared to 5-methylTHF and MeFox, as noted by the larger between-sample GCV and r_c.

In summary, these Round Robin studies for the measurement of serum folate forms by HPLC-MS/MS demonstrated the great value of conducting a systematic sample exchange with peer laboratories. Studies like this are an effective way to assess questions of laboratory comparability and method performance. It would be desirable if an external quality assessment scheme for the measurement of serum folate forms could be developed or if these analytes could be added to existing proficiency testing challenges that assess performance of total folate. Reference materials with certified concentrations for folate forms in addition to 5-methylTHF and for total folate are urgently needed to improve method accuracy. It was reassuring that the agreement among laboratories was better when the same method was used in different laboratories (group 2), but also that independently-developed methods can achieve similar results (group 1). This study also showed that errors in method calibration are a common source for inaccurate results. The reproducibility of a procedure in multiple laboratories and the comparability of different procedures are key requirements in the successful harmonization of biomarker measurements. This study is a first step towards that goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF 3rd, Mills JL, Pfeiffer CM, Fazili Z, Zhang M, Ueland PM, et al. Biomarkers of Nutrition for Development – Folate review. J Nutr. 2015; 145:1636S–80S. [PubMed: 26451605]
2. Gunter EW, Bowman BA, Caudill SP, Twite DB, Adams MJ, Sampson EJ. Results of an international round robin for serum and whole-blood folate. Clin Chem. 1996; 42:1689–94. [PubMed: 8855155]
3. Pfeiffer CM, Gunter EW, Caudill SP. Comparison of serum and whole blood folate measurements in 12 laboratories: an international study. Clin Chem. 2001; 47:A62. abstract 203.
4. Owen WE, Roberts WL. Comparison of five automated serum and whole blood folate assays. Am J Clin Pathol. 2003; 120:121–6. [PubMed: 12866382]
5. Pfeiffer, CM., Fazili, Z., Zhang, M. Folate analytical methodology. In: Bailey, LB., editor. Folate in Health and Disease. 2. Boca Raton: CRC Press, Taylor & Francis Group; 2010. p. 517-74.
6. Pfeiffer CM, Fazili Z, McCoy L, Zhang M, Gunter EW. Determination of folate vitamers in human serum by stable-isotope-dilution tandem mass spectrometry and comparison with radioassay and microbiologic assay. Clin Chem. 2004; 50:423–32. [PubMed: 14670827]
7. Fazili Z, Pfeiffer CM. Measurement of folates in serum and conventionally prepared whole blood lysates: application of an automated 96-well plate isotope-dilution tandem mass spectrometry method. Clin Chem. 2004; 50:2378–81. [PubMed: 15459090]
8. Fazili Z, Whitehead Ralph Jr, Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem. 2013; 405:4549–60. [PubMed: 23462981]
9. Fazili Z, Pfeiffer CM. Accounting for an isobaric interference allows correct determination of folate vitamers in serum by isotope dilution-liquid chromatography-tandem mass spectrometry. J Nutr. 2012; 143:108–13. [PubMed: 23173171]
10. Nelson BC, Pfeiffer CM, Margolis SA, Nelson CP. Solid-phase extraction-electrospray ionization mass spectrometry for quantification of folate in human plasma or serum. Anal Biochem. 2004; 325:41–51. [PubMed: 14715283]
11. Nelson BC, Satterfield MB, Sniegoski LT, Welch MJ. Simultaneous quantification of homocysteine and folate in human serum or plasma using liquid chromatography/tandem mass spectrometry. Anal Chem. 2005; 77:3586–93. [PubMed: 15924393]
12. Nelson BC, Pfeiffer CM, Margolis SA, Nelson CP. Affinity extraction combined with stable isotope dilution LC/MS for the determination of 5-methyltetrahydrofolate in human plasma. Anal Biochem. 2003; 313:117–27. [PubMed: 12576066]
13. Hart DJ, Finglas PM, Wolfe CA, Mellon F, Wright AJ, Southon S. Determination of 5-methyltetrahydrofolate (^{13}C-labeled and unlabeled) in human plasma and urine by combined liquid chromatography mass spectrometry. Anal Biochem. 2002; 305:206–13. [PubMed: 12054449]
14. Kok RM, Smith DE, Dainty JR, van den Akker JT, Finglas PM, Smulders YM, Jakobs C, de Meer K. 5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism. Anal Biochem. 2004; 326:129–38. [PubMed: 15003553]
15. Smith DE, Kok RM, Teerlink T, Jakobs C, Smulders YM. Quantitative determination of erythrocyte folate vitamer distribution by liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med. 2006; 44:450–9. [PubMed: 16599840]
16. Hannisdal R, Ueland PM, Svardal A. Liquid Chromatography-Tandem mass Spectrometry Analysis of folate and folate catabolites in human serum. Clin Chem. 2009; 55:1147–54. [PubMed: 19359539]
17. Mönch S, Netzel M, Netzel G, Rychlik M. Quantitation of folates and their catabolites in blood plasma, erythrocytes, and urine by stable isotope dilution assays. Anal Biochem. 2010; 398:150–60. [PubMed: 19903444]

18. Kirsch SH, Knapp J-P, Herrmann W, Obeid R. Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography-tandem mass spectrometry. J Chrom B. 2010; 878:68–75.

19. Kirsch SH, Herrmann W, Geisel J, Obeid R. Assay of whole blood (6S)-5-CH_3-H_4folate using ultra performance liquid chromatography tandem mass spectrometry. Anal Bioanal Chem. 2012; 404:895–902. [PubMed: 22729356]

20. Kiekens F, Van Daele J, Blancquaert D, Van Der Staeten D, Lambert WE, Stove CP. A validated ultra-high-performance liquid chromatography-tandem mass spectrometry method for the selective analysis of free and total folate in plasma and red blood cells. J Chrom A. 2015; 1398:20–28.

21. National Institute of Standards and Technology. [5 April 2017] Certificate of analysis, standard reference material 1955, homocysteine and folate in frozen human serum. Dec. 2015 Available from: https://www-s.nist.gov/srmors/view_cert.cfm?srm=1955

22. West A, Yan J, Perry C, Jiang X, Malysheva O, Caudill M. Folate-status response to a controlled folate intake in nonpregnant, pregnant, and lactating women. Am J Clin Nutr. 2012; 96:789–800. [PubMed: 22932279]

23. Satterfield MB, Sniegoski LT, Sharpless KE, Welch MJ, Hornikova A, Zhang N-F, Pfeiffer CM, Fazili Z, Zhang M, Nelson BC. Development of a new standard reference material: SRM 1955 (homocysteine and folate in human serum). Anal Bioanal Chem. 2006; 385:612–22. [PubMed: 16715281]

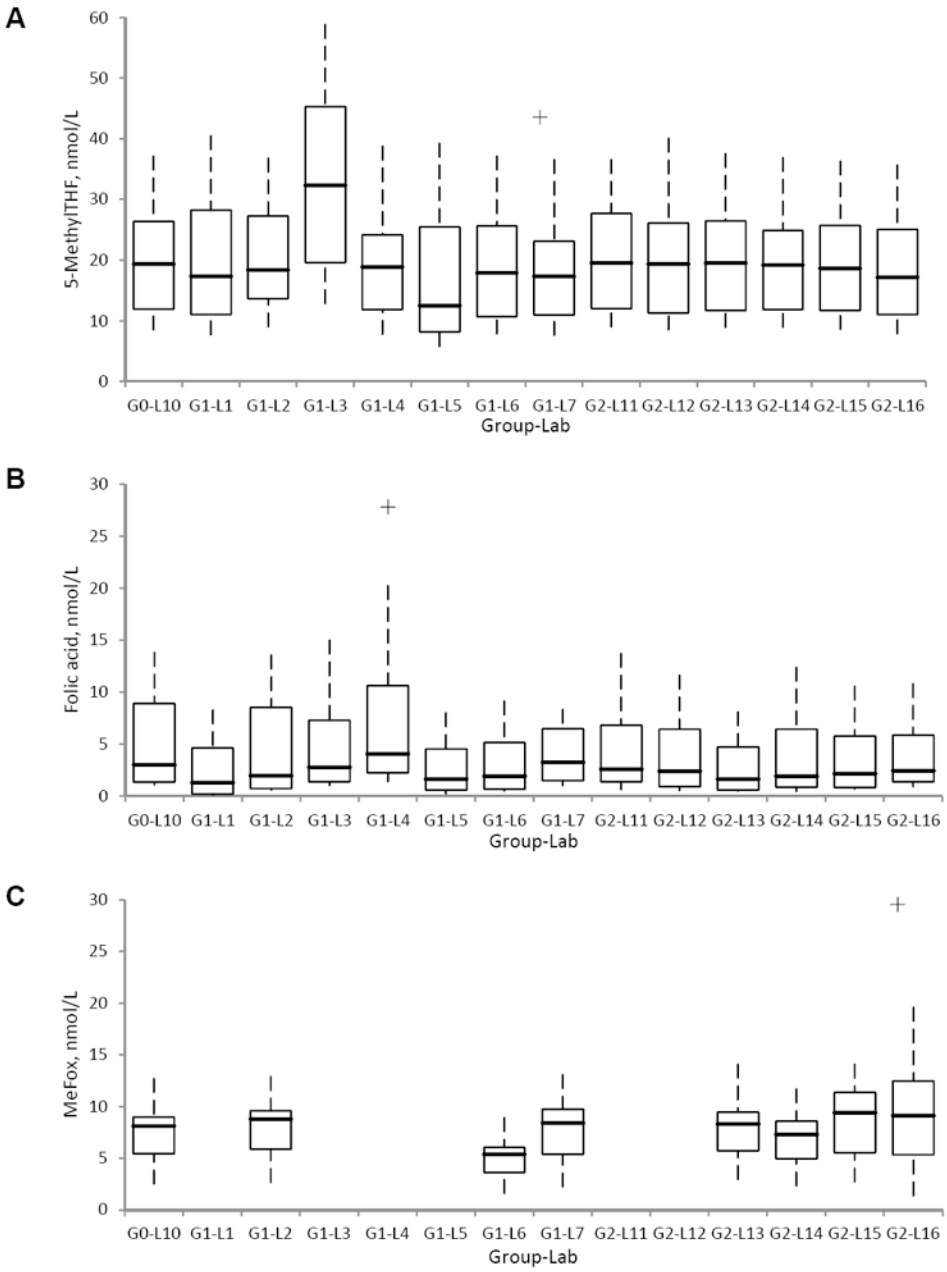

Figure 1.

Box-and-whisker plots of individually measured concentrations in 6 serum pools by group-laboratory for 5-methylTHF (panel A), folic acid (panel B), and MeFox (panel C). G0-L10 is the CDC laboratory; G1-L1 to G1-L7 are group 1 laboratories (used independently-developed HPLC-MS/MS methods); G2-L11 to G2-L16 are group 2 laboratories (used an adapted CDC method). Not all laboratories measured MeFox. The line and box represent the median and the 1st to 3rd quartiles, respectively; the whiskers are extending 1.5 * IQR from each quartile; observed values greater than 1.5 * IQR from each quartile are highlighted as possible near outliers. Each pool was measured in duplicate over 2 days, n = 4. 5-

MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF.

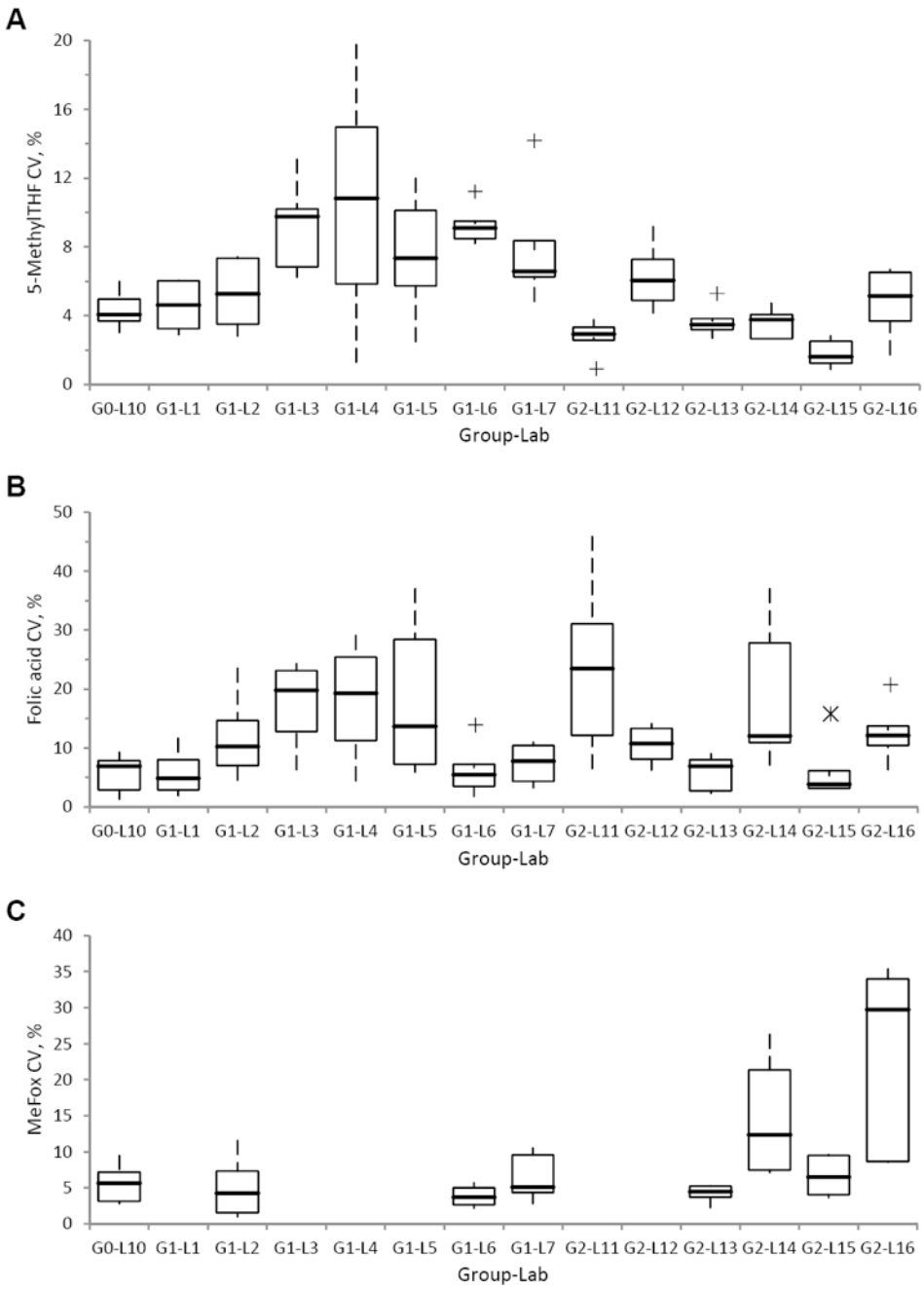

Figure 2.

Box-and-whisker plots of imprecision (CV) for 6 serum pools by group-laboratory for 5-methylTHF (panel A), folic acid (panel B), and MeFox (panel C). G0-L10 is the CDC laboratory; G1-L1 to G1-L7 are group 1 laboratories (used independently-developed HPLC-MS/MS methods); G2-L11 to G2-L16 are group 2 laboratories (used an adapted CDC method). Not all laboratories measured MeFox. The line and box represent the median and the 1st to 3rd quartiles, respectively; the whiskers are extending 1.5 * IQR from each quartile; observed values greater than 1.5 * IQR from each quartile are highlighted as possible near (+) or far (∗) outliers. Each pool was measured in duplicate over 2 days, n = 4.

5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF.

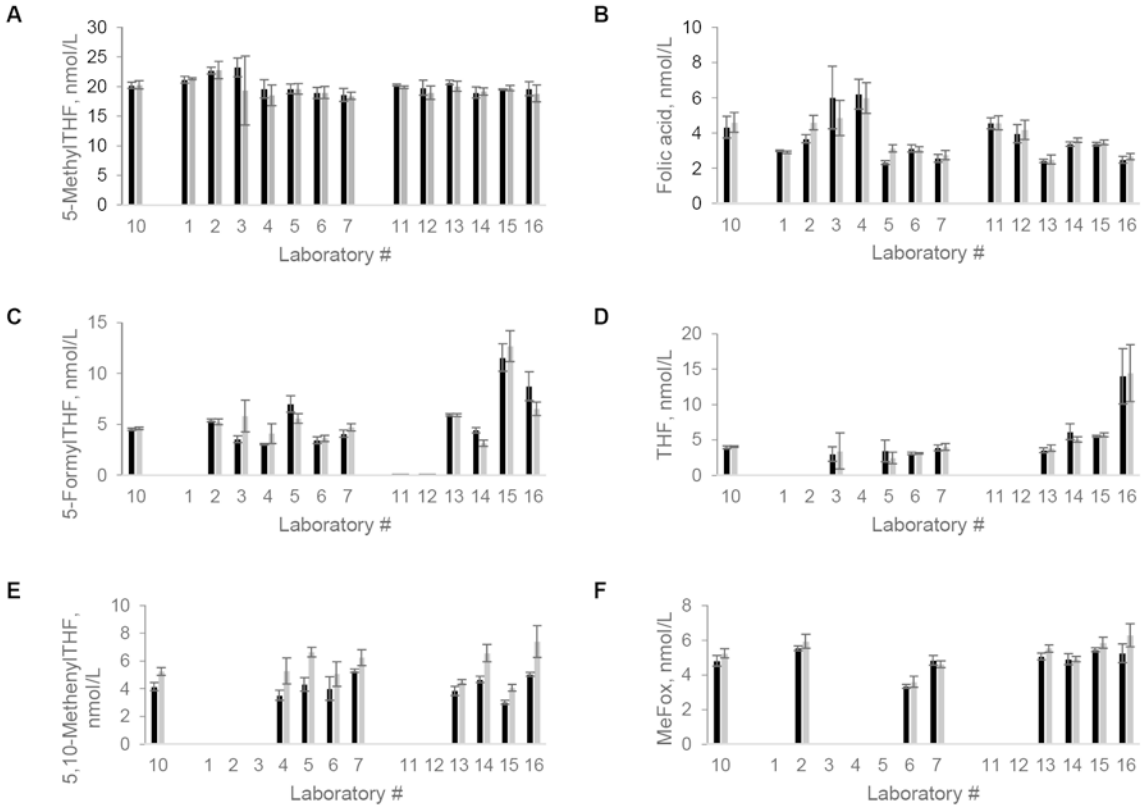

Figure 3.

Mean concentrations measured in CDC folate calibrators for 5-methylTHF (panel A), folic acid (panel B), 5-formylTHF (panel C), THF (panel D), 5,10-methenylTHF (panel E), and MeFox (panel F). Black bars represent single calibrators; grey bars represent mixed calibrators; error bars represent SD. Laboratory #10 is the CDC laboratory; laboratories #1–#7 (group 1) used independently-developed HPLC-MS/MS methods; laboratories #11–#16 (group 2) used an adapted CDC method. Not all laboratories measured 5-formylTHF, THF, 5,10-methenylTHF, and MeFox. Each calibrator was measured in duplicate over 2 days, n = 4. 5-Methyltetrahydrofolate, 5-methylTHF, 5-formylTHF, 5-formyltetrahydrofolate; THF, tetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; and MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF.

Table 1

HPLC-MS/MS methods used by Round Robin participants to measure serum folate forms[1]

Laboratory #	Affiliation	Sample extraction	Calibrator extraction[2]	Chromatography	Mass spectrometer	Folates measured[3]	Reference
10	CDC, USA	SPE (RP-Phenyl)	Yes	RP-C8, isocratic	AB Sciex 5500	A–F	6, 8
Group 1							
1	Tufts University, USA	Deproteination (MeCN), evaporation, reconstitution	Yes	RP-C8, gradient	AB Sciex QTrap 5500	A, B	16
2	Bevital, Norway	Deproteination (MeCN), evaporation, reconstitution	Yes	RP-C8, gradient	AB Sciex 4000	A–D	16
3	Technical University of Munich, Germany	SPE (SAX)	No	RP-C18, gradient	Thermo Finnigan TSQ Quantum Discovery	A, B, D, E	17
4	VU University Medical Center, Amsterdam, Netherlands	Heat extraction, affinity chromatography	No	RP-C18, gradient	AB Sciex 5500	A, B, D–F	14, 15
5	Saarland University, Germany	SPE (MAX)	Yes	RP-C18, gradient	Waters Micromass Quattro Premier Xe	A, B, D–F	18, 19
6	Roche, Germany	SPE (RP-Phenyl)	Yes	RP-C18, gradient	Thermo Finnigan TSQ Quantum Ultra	A–F	In-house method
7	Ghent University, Belgium	Deproteination (acid), SPE (RP-C18)	Yes	RP-C18, gradient	AB Sciex 4000	A–F	20
Group 2							
11	Cornell University, USA	SPE (RP-Phenyl)	Yes	RP-C18, gradient	Thermo Finnigan TSQ Quantum Access	A, B	6, 22
12	University of Toronto, Canada	SPE (RP-Phenyl)	Yes	RP-C18 PFP, gradient	AB Sciex 5500	A, B	6, modified
13	University of British Columbia, Canada	SPE (RP-Phenyl)	Yes	RP-C8, isocratic	AB Sciex 4000	A–F	6, 8
14	MRC Cambridge, UK	SPE (RP-Phenyl)	Yes	RP-C8, isocratic	AB Sciex 4000	A–F	6
15	University of Otago, New Zealand	SPE (RP-Phenyl)	Yes	RP-C8, isocratic	AB Sciex 3200	A–F	6, 8
16	NIST, USA	SPE (RP-Phenyl)	Yes	RP-C8, isocratic	Agilent 6460	A–F	6, 8

[1] MAX, mixed-mode anion exchange; MeCN, acetonitrile; PFP, pentafluorophenyl; RP, reversed phase; SAX, strong anion exchange; SPE, solid-phase extraction.

[2] Yes, calibrators subjected to all sample extraction steps; No, calibrators measured directly.

[3] A, 5-methyltetrahydrofolate; B, folic acid; C, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; D, 5-formyltetrahydrofolate; E, tetrahydrofolate; F, 5,10-methenyltetrahydrofolate.

Table 2

Concentration and imprecision for 5-methyltetrahydrofolate by laboratory and serum pool[1]

	Serum pool concentrations, nmol/L						Serum pool CV, %						Median
Laboratory #	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**CV, %**
10	9.21	15.2	12.0	23.8	26.4	35.7	6.0	4.1	4.9	4.0	3.0	3.7	4.1
Group 1													
1	8.20	13.6	10.9	22.4	28.2	38.9	5.2	4.0	6.0	6.1	2.9	3.3	4.6
2	9.41	14.5	14.2	22.3	27.4	34.8	2.8	7.3	4.6	3.6	7.4	5.9	5.3
3	13.5	24.8	19.8	37.3	46.0	51.9	9.9	13	9.9	6.2	6.9	9.6	9.8
4	9.34	13.8	11.4	21.6	24.6	36.8	20	15	12	1.3	9.6	6.3	11
5	5.91	9.60	8.30	15.9	26.3	36.3	2.5	7.4	12	7.2	10	6.0	7.3
6	8.62	13.8	10.9	22.4	24.7	33.4	9.4	8.5	8.2	9.0	9.1	11	9.1
7	8.48	13.1	11.0	21.9	22.5	36.3	7.8	6.4	6.6	4.8	6.5	14	6.6
Mean	*9.07*	*14.7*	*12.4*	*23.4*	*28.5*	*38.3*	*8.2*	*8.7*	*8.5*	*5.5*	*7.5*	*8.1*	
SD	*2.28*	*4.72*	*3.70*	*6.56*	*7.94*	*6.22*	*5.9*	*3.7*	*2.9*	*2.5*	*2.4*	*3.8*	
Group 2													
11	9.30	15.2	12.0	24.6	27.4	35.7	2.7	3.1	0.9	3.8	3.3	2.7	2.9
12	9.00	15.6	11.4	24.4	26.3	37.4	6.2	5.0	7.1	5.8	4.2	9.2	6.0
13	9.24	14.7	11.6	24.3	26.4	36.0	3.7	5.3	3.2	2.7	3.5	3.5	3.5
14	9.14	14.7	11.8	24.0	25.1	35.5	2.7	3.6	4.7	3.9	4.0	2.7	3.8
15	8.86	14.7	11.7	23.3	25.6	35.7	2.5	0.9	1.3	2.8	1.2	2.0	1.7
16	8.41	13.4	11.1	21.4	24.9	34.0	6.2	1.7	4.1	6.5	6.7	3.9	5.2
Mean	*8.99*	*14.7*	*11.6*	*23.7*	*26.0*	*35.7*	*4.0*	*3.3*	*3.6*	*4.3*	*3.8*	*4.0*	
SD	*0.33*	*0.74*	*0.32*	*1.20*	*0.94*	*1.09*	*1.8*	*1.7*	*2.3*	*1.6*	*1.8*	*2.6*	
Overall mean[2]	*9.04*	*14.8*	*12.0*	*23.5*	*27.3*	*37.0*	*6.2*	*6.1*	*6.1*	*4.8*	*5.6*	*6.0*	
SD	*1.56*	*3.24*	*2.55*	*4.52*	*5.58*	*4.49*	*4.7*	*3.9*	*3.5*	*2.1*	*2.8*	*3.7*	

[1] Concentration values are means, n = 4 (i.e., duplicate analysis on 2 days); CV values were calculated as (pool SD/pool mean * 100); serum pool 6 was same material as serum pool 5 except that serum pool 6 was spiked with 10 nmol/L 5-methyltetrahydrofolate and 5 nmol/L each of folic acid, 5-formyltetrahydrofolate, tetrahydrofolate, 5,10-methenyltetrahydrofolate, and pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate.

[2] Includes results from laboratory #10.

Table 3

Concentration and imprecision for folic acid by laboratory and serum pool[1]

	Serum pool concentration, nmol/L						Serum pool CV, %						Median
Laboratory #	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**CV, %**
10	1.21	2.11	1.28	4.10	8.82	13.6	9.3	7.7	6.2	7.6	3.0	1.3	6.9
10[2]	0.69	1.32	0.76	2.75	5.61	9.43	0.5	3.2	5.3	1.3	2.4	1.6	2.0
Group 1													
1	0.21	0.61	0.17	2.03	4.77	8.12	7.7	4.1	12	1.9	5.6	3.0	4.9
2	0.70	1.26	0.73	3.56	8.45	12.8	8.0	14	12	23	4.5	7.3	10
3	1.30	1.88	1.30	4.23	7.45	11.7	6.3	17	24	23	13	23	20
4	1.85	2.91	1.95	5.45	10.6	20.5	29	14	25	12	4.4	25	19
5	0.42	0.76	0.26[3]	1.73	4.22	7.55	37	28	14	7.4	13	5.9	14
6	0.61	1.26	0.70	2.57	5.20	8.83	14	4.7	6.7	1.8	6.2	3.7	5.5
7	<LOD	1.11	<LOD	2.05	4.48	8.12	n/a[4]	9.6	n/a[4]	11	5.9	3.3	7.8
Mean	*0.85*	*1.40*	*0.85*	*3.09*	*6.45*	*11.1*	*17*	*13*	*16*	*11*	*7.6*	*10*	
SD	*0.61*	*0.78*	*0.67*	*1.38*	*2.43*	*4.60*	*13*	*8.1*	*7.3*	*8.9*	*3.9*	*9.6*	
Group 2													
11	1.10	1.82	1.19	3.53	6.93	12.0	46	30	30	17	6.5	13	23
12	0.70	1.52	0.92	3.33	6.33	10.6	14	13	6.2	12	8.3	9.7	11
13	0.55	1.07	0.55	2.27	4.72	7.93	7.9	9.0	7.6	6.2	2.3	2.8	6.9
14	0.63	1.34	0.83	2.48	6.44	10.5	37	27	11	11	7.1	13	12
15	0.74	1.47	0.80	2.90	5.86	10.1	16	3.2	4.3	5.3	3.5	3.1	3.9
16	1.34	1.93	1.18	3.18	5.84	9.16	12	13	21	11	6.3	13	12
Mean	*0.84*	*1.53*	*0.91*	*2.95*	*6.02*	*10.1*	*22*	*16*	*13*	*10*	*5.7*	*8.9*	
SD	*0.31*	*0.31*	*0.24*	*0.49*	*0.75*	*1.39*	*16*	*10*	*9.9*	*4.3*	*2.3*	*4.8*	
Overall mean[3]	*0.83*	*1.45*	*0.87*	*3.00*	*6.21*	*10.5*	*18*	*13*	*14*	*10*	*6.4*	*9.0*	
SD	*0.44*	*0.57*	*0.46*	*0.99*	*1.74*	*3.30*	*14*	*9.1*	*8.4*	*7.1*	*3.4*	*7.5*	

[1]Concentration values are means, n = 4 (i.e., duplicate analysis on 2 days); CV values were calculated as (pool SD/pool mean * 100); serum pool 6 was same material as serum pool 5 except that serum pool 6 was spiked with 10 nmol/L 5-methyltetrahydrofolate and 5 nmol/L each of folic acid, 5-formyltetrahydrofolate, tetrahydrofolate, 5,10-methenyltetrahydrofolate, and pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate.

[2]Folic acid results after laboratory 10 corrected folic acid calibration bias; samples were analyzed in 1 replicate on 2 days.

[3] Result is from duplicate analysis on day 1; day 2 results were <LOD (<0.5 nmol/L).

[4] n/a, no CV calculated because laboratory only provided results <LOD (<0.33 nmol/L).

[2] Includes results from laboratory #10 after correcting folic acid calibration bias.

Table 4

Concentration and imprecision for MeFox by laboratory and serum pool[1]

	Serum pool concentration, nmol/L						Serum pool CV, %						Median
Laboratory #	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**CV, %**
10	5.43	9.10	8.30	12.0	2.63	7.76	9.5	6.2	2.7	7.0	3.2	5.0	5.6
Group 1													
2	6.04	9.57	9.32	12.5	2.90	8.73	12	1.6	4.2	4.2	6.9	0.9	4.2
6	3.67	6.10	5.84	8.63	1.79	5.09	2.2	3.8	4.9	3.6	5.7	2.7	3.7
7	5.46	9.57	9.03	12.0	2.45	7.92	4.4	5.1	5.1	9.5	11	2.8	5.1
Mean	*5.06*	*8.41*	*8.06*	*11.0*	*2.38*	*7.25*	*6.1*	*3.5*	*4.7*	*5.8*	*7.7*	*2.1*	
SD	*1.24*	*2.00*	*1.93*	*2.10*	*0.56*	*1.91*	*4.9*	*1.8*	*0.5*	*3.2*	*2.5*	*1.1*	
Group 2													
13	5.64	9.50	8.66	13.5	3.08	8.15	5.3	4.3	3.8	4.6	5.2	2.2	4.5
14	5.24	8.76	7.52	8.79	2.76	8.44	21	7.5	7.1	13	12	26	12
15	5.69	11.4	9.71	13.4	3.01	8.95	7.9	3.6	4.1	5.0	9.6	9.5	6.5
16	5.75	11.0	13.0	19.9	2.90	7.38	8.7	8.6	30	34	35	29	30
Mean	*5.58*	*10.2*	*9.72*	*13.9*	*2.94*	*8.23*	*11*	*6.0*	*11*	*14*	*16*	*17*	
SD	*0.23*	*1.24*	*2.36*	*4.57*	*0.14*	*0.66*	*7.0*	*2.4*	*13*	*14*	*14*	*13*	
Overall mean[2]	*5.37*	*9.38*	*8.92*	*12.6*	*2.69*	*7.80*	*8.8*	*5.1*	*7.8*	*10*	*11*	*9.8*	
SD	*0.73*	*1.60*	*2.05*	*3.50*	*0.42*	*1.21*	*5.7*	*2.3*	*9.2*	*10*	*10*	*11*	

[1] Concentration values are means, n = 4 (i.e., duplicate analysis on 2 days); CV values were calculated as (pool SD/pool mean * 100); laboratories #1, #3, #4, #5, #11, and #12 did not measure MeFox; serum pool 6 was same material as serum pool 5 except that serum pool 6 was spiked with 10 nmol/L 5-methyltetrahydrofolate and 5 nmol/L each of folic acid, 5-formyltetrahydrofolate, tetrahydrofolate, 5,10-methenyltetrahydrofolate, and pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate.

[2] Includes results from laboratory #10.

Table 5

Concentration and imprecision for 3 minor folate forms in a spiked serum sample[1]

	Concentration, nmol/L			CV, %		
Laboratory #	**5-FormylTHF**	**THF**	**5,10-MethenylTHF**	**5-FormylTHF**	**THF**	**5,10-MethenylTHF**
10	5.21	5.42	4.84	6.9	4.3	4.7
Group 1						
1	Not measured	Not measured	Not measured	Not measured	Not measured	Not measured
2	6.02	Not measured	Not measured	15	Not measured	Not measured
3	5.13	6.78	Not measured	22	38	Not measured
4	4.86	<LOD[2]	5.29	10	n/a	9.2
5	7.01	4.63	5.06	4.9	5.8	10
6	4.41	5.39	4.97	7.5	7.3	19
7	4.68	4.38	5.79	4.0	11	4.8
Mean	*5.35*	*5.30*	*5.28*	*11*	*16*	*11*
SD	*0.98*	*1.08*	*0.37*	*6.9*	*15*	*6.0*
Group 2						
11	Not measured	Not measured	Not measured	Not measured	Not measured	Not measured
12	Not measured	Not measured	Not measured	Not measured	Not measured	Not measured
13	6.65	5.05	4.17	5.7	2.1	6.1
14	4.61	7.20	5.26	7.9	11	3.8
15	12.9	7.43	3.63	4.2	5.9	5.6
16	11.0	24.1	3.95	7.6	4.9	13
Mean	*8.79*	*10.9*	*4.25*	*6.4*	*6.0*	*7.1*
SD	*3.82*	*8.84*	*0.71*	*1.7*	*3.7*	*4.0*

[1]Concentration values are means, n = 4 (i.e., duplicate analysis on 2 days); CV values were calculated as (pool SD/pool mean * 100); sample was spiked with 10 nmol/L 5-methyltetrahydrofolate and 5 nmol/L each of folic acid, 5-formyltetrahydrofolate (5-formylTHF), tetrahydrofolate (THF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), and pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate; LOD, limit of detection.

[2]LOD: <0.5 nmol/L

Table 6

Random effects model to assess contribution of sample variation and analytic variation to total variation[1]

					Analytic variation				
			Between-sample		Between-day		Within-day		
Analyte	Laboratory	Geometric mean, nmol/L	GCV, %	% of total variance	GCV, %	% of total variance	GCV, %	% of total variance	Repeatability coefficient[2] (r_c)
5-MethylTHF	10	18.3	55.3	99.0	4.8	0.9	1.9	0.1	1.06
	1	17.6	65.5	99.4	0.0	0.0	4.8	0.6	1.14
	2	18.6	51.3	98.7	0.0	0.0	5.6	1.3	1.17
	3	28.9	56.0	96.9	0.0	0.0	9.4	3.1	1.30
	4	17.4	55.8	94.2	8.5	2.5	9.7	3.2	1.31
	5	13.8	80.0	98.6	3.4	0.2	7.5	1.1	1.23
	6	16.9	56.2	95.6	10.9	4.1	3.1	0.3	1.09
	7	16.7	58.0	97.7	2.5	0.2	7.9	2.1	1.25
	11	18.6	56.0	99.7	0.0	0.0	2.9	0.3	1.08
	12	18.4	58.5	98.6	0.0	0.0	6.5	1.4	1.20
	13	18.2	56.7	99.5	0.0	0.0	3.7	0.5	1.11
	14	18.0	55.4	99.5	0.9	0.0	3.6	0.5	1.10
	15	17.9	56.6	99.9	0.0	0.0	1.9	0.1	1.05
	16	16.8	57.2	98.9	3.8	0.5	4.1	0.6	1.12
PGA	10	3.42	133	99.5	3.8	0.1	5.7	0.3	1.17
	1	1.09	361	99.8	3.8	0.1	5.9	0.1	1.18
	2	2.49	196	98.7	5.8	0.2	13.0	1.0	1.43
	3	3.20	117	95.7	11.5	1.4	16.1	2.8	1.56
	4	4.74	126	94.8	20.0	3.9	11.3	1.3	1.37
	5	1.46	229	97.3	13.9	1.0	17.8	1.7	1.63
	6	1.99	151	99.5	1.8	0.0	7.3	0.4	1.22
	7	3.01	107	99.2	0.0	0.0	8.0	0.8	1.25
	11	2.90	131	90.5	29.0	7.3	15.5	2.2	1.53
	12	2.45	150	98.9	6.0	0.3	10.0	0.8	1.32
	13	1.74	158	99.7	0.0	0.0	6.5	0.3	1.20
	14	2.17	163	96.3	12.9	1.2	18.5	2.5	1.66

			Analytic variation						
			Between-sample		Between-day		Within-day		
Analyte	Laboratory	Geometric mean, nmol/L	GCV, %	% of total variance	GCV, %	% of total variance	GCV, %	% of total variance	Repeatability coefficient[2] (r_c)
	15	2.30	147	99.6	0.0	0.0	7.0	0.4	1.22
	16	2.81	98.6	97.5	0.0	0.0	13.3	2.5	1.44
MeFox	10	6.80	57.0	98.3	6.1	1.3	3.5	0.4	1.10
	2	7.43	55.5	98.3	5.0	0.9	4.5	0.7	1.13
	6	4.66	58.7	99.3	3.5	0.4	2.8	0.3	1.08
	7	6.91	62.1	98.5	2.4	0.2	6.5	1.3	1.20
	13	7.35	54.5	99.3	0.0	0.0	4.4	0.7	1.13
	14	6.37	47.6	88.5	8.9	3.4	13.7	8.1	1.46
	15	7.79	59.3	98.3	3.2	0.3	6.6	1.4	1.20
	16	8.15	75.4	83.7	0.0	0.0	30.2	16.3	2.27

[1] Two-way random effects model with sample and day of analysis as random effects; between-sample, segment due to variation between samples; analytic variation, segment due to repeated measurements; 6 serum pools were analyzed in 4 replicates (duplicate analysis on 2 days).

[2] Repeatability coefficient r_C is the anti-log of the traditional repeatability coefficient RC and expresses the 95% range for the ratio between 2 replicates on the same day (i.e. ratio / e^{RC}, ratio * e^{RC}), where $RC = \sqrt{2} * 1.96 * SD_{log}$ and SD_{log} was the within-day SD estimated from the model.

Table 7

Recovery of 6 folate forms spiked into a serum sample[1]

	Recovery ± SD, %					
Laboratory #	**5-MethylTHF**	**PGA**	**MeFox**	**5-FormylTHF**	**THF**	**5,10-MethenylTHF**
10	93 ± 5.6	95 ± 2.9	103 ± 6.1	104 ± 7.2	101 ± 4.6	97 ± 4.5
Group 1						
1	107 ± 12	67 ± 5.4	Not measured	Not measured	Not measured	Not measured
2	75 ± 37	87 ± 26	117 ± 2.4	120 ± 18	Not measured	Not measured
3	59 ± 68	85 ± 46	Not measured	103 ± 22	136 ± 38	Not measured
4	122 ± 27	198 ± 98	No measured	97 ± 9.7	0	106 ± 9.7
5	100 ± 5.3	67 ± 15	Not measured	140 ± 6.8	93 ± 5.0	101 ± 11
6	87 ± 23	73 ± 7.7	66 ± 1.8	88 ± 6.6	108 ± 7.8	99 ± 19
7	138 ± 53	73 ± 5.3	109 ± 9.2	94 ± 3.7	88 ± 9.4	116 ± 5.6
Mean ± SD	*98 ± 27*	*93 ± 47*	*97 ± 27*	*107 ± 20*	*85 ± 51*	*106 ± 7.3*
Group 2						
11	82 ± 13	102 ± 25	Not measured	Not measured	Not measured	Not measured
12	111 ± 43	85 ± 17	Not measured	Not measured	Not measured	Not measured
13	96 ± 21	64 ± 3.0	101 ± 6.3	133 ± 7.6	91 ± 5.7	83 ± 5.1
14	104 ± 4.2	81 ± 34	114 ± 40	92 ± 7.3	129 ± 28	105 ± 4.0
15	101 ± 7.0	86 ± 9.5	119 ± 13	259 ± 11	145 ± 3.0	73 ± 4.0
16	91 ± 6.1	66 ± 5.1	90 ± 15	221 ± 17	460 ± 31	79 ± 11
Mean ± SD	*98 ± 10*	*81 ± 14*	*106 ± 13*	*176 ± 77*	*206 ± 171*	*85 ± 14*

[1]Values are means ± SD, n = 4 (i.e., duplicate analysis on 2 days); serum sample was spiked with 10 nmol/L 5-methyltetrahydrofolate (5-methylTHF) and 5 nmol/L each of folic acid (PGA), 5-formyltetrahydrofolate (5-formylTHF), tetrahydrofolate (THF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), and pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF (MeFox).

Table 8

Concentrations of 5-methyltetrahydrofolate and folic acid in NIST SRM 1955 by laboratory[1]

	5-Methyltetrahydrofolate				Folic acid			
	Concentration ± SD, nmol/L			Mean relative bias ± SD[2], %	Concentration ± SD, nmol/L			Mean relative bias ± SD[3], %
Laboratory #	Level 1	Level 2	Level 3		Level 1	Level 2	Level 3	
10	5.01 ± 0.03	11.0 ± 0.28	38.9 ± 0	12 ± 5.9	0.86 ± 0.02	1.95 ± 0.11	1.70 ± 0	73 ± 13
10[4]	n/a	n/a	n/a	n/a	0.54 ± 0.02	1.17 ± 0.02	1.14 ± 0.05	9.4 ± 3.9
Group 1								
1	4.88 ± 0.14	9.98 ± 1.31	37.0 ± 0.94	5.6 ± 9.4	0.30 ± 0.02	0.49 ± 0.01	0.54 ± 0.08	-47 ± 7.8
2	5.24 ± 0.17	12.1 ± 0.71	39.7 ± 1.84	18 ± 9.7	0.82 ± 0.02	1.08 ± 0.06	0.97 ± 0.04	20 ± 37
3	5.65 ± 1.63	13.6 ± 3.98	44.2 ± 2.47	30 ± 27	5.50 ± 1.27	4.70 ± 1.41	4.95 ± 0.92	578 ± 371
4	4.10 ± 0.50	9.00 ± 0.51	37.8 ± 2.72	-3.6 ± 8.0	2.15 ± 0.27	2.69 ± 0.32	3.11[5]	236 ± 100
5	3.69 ± 0.26	6.69 ± 0.69	26.6 ± 1.49	-24 ± 9.7	0.53 ± 0.31	0.48 ± 0.01	0.49 ± 0.12	-33 ± 44
6	4.16 ± 0.03	9.27 ± 0.02	35.8 ± 0.66	-3.5 ± 1.4	0.58 ± 0.01	1.07 ± 0.04	1.04 ± 0.03	5.8 ± 10
7	5.14 ± 0.27	10.1 ± 0.32	38.0 ± 0.83	9.0 ± 9.7	<LOD	1.06 ± 0.01	1.08 ± 0.02	0.7 ± 1.4
Mean ± SD	*4.69 ± 0.72*	*10.1 ± 2.23*	*37.0 ± 5.33*		*1.65 ± 2.00*	*1.65 ± 1.53*	*1.74 ± 1.67*	
Group 2								
11	4.79 ± 0.23	10.3 ± 0.05	37.5 ± 1.56	6.6 ± 5.9	0.53 ± 0.03	1.14 ± 0.06	1.36 ± 0.01	15 ± 10
12	4.74 ± 0.25	11.5 ± 0.83	44.3 ± 2.92	16 ± 6.9	0.67 ± 0.04	1.31 ± 0.05	1.52 ± 0.19	35 ± 12
13	4.43 ± 0.07	9.80 ± 0.19	38.6 ± 0.32	2.9 ± 2.1	0.47 ± 0.02	0.98 ± 0.02	1.07 ± 0.05	-3.3 ± 4.2
14	4.19 ± 0.23	10.3 ± 0	38.2 ± 0.64	2.3 ± 4.3	0.43 ± 0	1.02 ± 0.11	1.15 ± 0.01	-2.5 ± 10
15	4.51 ± 0.08	10.3 ± 0.07	37.6 ± 0.07	4.1 ± 2.4	0.54 ± 0.12	1.24 ± 0.03	1.28 ± 0.04	16 ± 11
16	4.48 ± 0.28	9.36 ± 0.41	35.9 ± 0.25	-0.6 ± 5.7	0.95 ± 0.02	1.62 ± 0.19	1.65 ± 0.24	67 ± 24
Mean ± SD	*4.52 ± 0.22*	*10.3 ± 0.72*	*38.7 ± 2.90*		*0.60 ± 0.19*	*1.22 ± 0.23*	*1.34 ± 0.22*	

[1] Values are means ± SD, n = 2 (i.e., duplicate analysis).

[2] Mean relative bias represents percent deviation from certified value (level 1, 2, 3: 4.26, 9.73, and 37.1 nmol/L, respectively).

[3] Mean relative bias represents percent deviation from reference value (level 1, 2, 3: 0.49, 1.05, and 1.07 nmol/L, respectively).

[4] Folic acid results after laboratory 10 corrected folic acid calibration bias; n/a, 5-methyltetrahydrofolate results not re-measured.

[5] Laboratory provided 1 result >LOD and 1 result <LOD (<0.5 nmol/L); result <LOD was not used to calculate the mean.